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14. ABSTRACT
This project proposes novel and unbiased genomic approaches to identify genes associated with salamander limb regeneration. The first objective will extend an ongoing study of the transcriptional basis of limb regeneration in the Mexican axolotl (Ambystoma mexicanum) to three additional salamander species (A. tigrinum, A. maculatum, and Notophthalmus viridescens). Transcripts will be sampled at four times during limb regeneration to characterize temporal changes in abundance that coincide with wound healing and blastema formation. Accomplishment of this objective will enhance an ongoing DOD Multi-Investigator Research Initiative by resolving genes and molecular

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Stephen Voss
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Report Title

Final Report: Application of Comparative Functional Genomics to Identify Regeneration-Specific Genes

ABSTRACT

This project proposes novel and unbiased genomic approaches to identify genes associated with salamander limb regeneration. The first objective will extend an ongoing study of the transcriptional basis of limb regeneration in the Mexican axolotl (Ambystoma mexicanum) to three additional salamander species (A. tigrinum, A. maculatum, and Notophthalmus viridescens). Transcripts will be sampled at four times during limb regeneration to characterize temporal changes in abundance that coincide with wound healing and blastema formation. Accomplishment of this objective will enhance an ongoing DOD Multi-Investigator Research Initiative by resolving genes and molecular pathways for translation to mammalian injury models. The second objective will use quantitative trait locus (QTL) mapping and a newly derived salamander line to identify genes that contribute to variation in limb regeneration. Genotypic and statistical approaches will be used to identify genotype-phenotype associations indicative of QTL for regeneration traits. Accomplishment of this objective will set the stage for identifying loci that explain genetic and developmental sources of variation during limb regeneration. Overall, the project is significant because novel approaches will be used to reveal fundamental regenerative processes that could be engineered in humans to dramatically improve the quality and length of life after injury.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received	Book Chapter
08/22/2014	1.00 Stephen R Voss, Antony Athippozhy, Ryan M. Woodcock. Transcriptomics using axolotls, Molecular Methods: Springer, (01 2015)
TOTAL:	**1**

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Nour Al Haj Baddar	0.25	
Alex Palumbo	0.25	
FTE Equivalent:	**0.50**	
Total Number:	**2**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Stephen R Voss	0.05	
FTE Equivalent:	**0.05**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Gareth Voss	0.30	
FTE Equivalent:	**0.30**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Please see attachment below.

Technology Transfer

This project had two primary objectives: (1) Perform a comparative analysis of limb regeneration transcriptomes for 4 salamander species, and (2) Perform a quantitative genetic analysis of limb regeneration using hybrid salamanders. These objectives are novel and designed to identify regeneration-associated genes.

Accomplishments: We accomplished the first objective during the final funding period. In review, we collected 54 tissue samples from regenerating limbs of *A. mexicanum, A. andersoni,* and *A. maculatum.* We performed RNA-seq for 36 samples (12 from each species) and then used the 18 samples from *A. mexicanum* as technical replicates for microarray analysis. In the past year, we developed an RNA-seq bioinformatic schema to re-analyze the data in a way that best leverages existing transcript resources for *A. mexicanum.* During the last 5 years, the *A. mexicanum* transcriptome has been deep sequenced. However, there are no existing transcript data for *A. andersoni,* and *A. maculatum.* Thus, we first built a reference transcriptome for *A. mexicanum,* using all available data at Sal-Site and the data generated from this experiment, and then used this as a model for annotating contigs that were generated for *A. andersoni* and *A. maculatum.* Additionally, we built a separate data partition for all 1:1 matches between *A. mexicanum* RNA-seq contigs and probe-sets on the *A. mexicanum* Affymetrix GeneChip. This later dataset will be used to specifically compare results between the two gene expression platforms. This reanalysis of the data wis necessary, but it does set back slightly the completion date for manuscripts arising from this work, which should occur before the end of 2014. We did in the last year commit effort to writing a book chapter for an upcoming Regeneration Methods book, to be published by Wiley. Our chapter entitled *Transcriptomics using the axolotl* is based upon the experience that we gained under this funding mechanism and a MURI grant that comprehensively detailed gene expression during the first 28 days of limb regeneration. In our chapter, we describe best practices for performing gene expression analyses using either the Affymetrix GeneChip or RNA-seq. The information contained in this chapter will be an important deliverable to the regeneration community.

The second objective of this project was to perform a limb amputation experiment and then pursue QTL analysis to identify genes that explain variation in rate of limb regeneration. Early in this project we completed a QTL analysis using adult axolotls and a tail regeneration model. The results from this study were published in *PlosOne* and other results from this experiment were published in *Heredity.* This past year, we were unable to make a hybrid cross to perform the limb regeneration QTL analysis…it is not always straightforward to mate different salamander species using *in vitro* fertilization. Thus, we turned our attention to developing a new axolotl model for regenerative biology. As it turned out, this was an incredibly smart decision. Below, I detail some of our early results that justify a grant submission to obtain funding to further exploit this model.

One of the biggest limitations in working with highly regenerative salamander models is the time it takes to rear them to juvenile and adult stages. Developmental rate is longer for axolotls than *Xenopus* and *Danio,* which evolved in warmer

climates. The axolotl embryonic period spans 20-25 days and sexual maturity is reached after 1-1.5 years. There is need for an early stage, axolotl regeneration model that would increase the pace of experimentation, increase efficiency of animal use, and allow more robust experimental designs. While regeneration assays have been established for early stages of the *Xenopus* and *Danio*, no comparable assays have been established for the highly regenerative Mexican axolotl. Conceptually, the development of an early stage assay for the axolotl would reveal the extent to which regeneration programs are conserved among these disparate, highly regenerative models. While it seems likely that some conserved signaling pathways are similar, novel regenerative mechanisms maybe unique to these evolutionarily distant, non-mammalian vertebrates.

We developed an early stage tail regeneration assay using axolotl embryos. There are several advantages of this model. First, the assay uses ~20 day old (i.e. post fertilization) embryos that have sufficient yolk reserves to fully regenerate an amputated, distal tail tip in 7 days. The approximately 1 cm embryos are efficiently reared in microtiter plates with out need for feeding. At this stage of development, axolotl embryos do not have capacity to mount an adaptive immune response and the innate immune response is immature. The immature state of the axolotl embryo immune system is hugely advantageous for interpreting gene expression patterns from heterogeneous tissue samples. Generally, the innate immune response involves activation of phagocytic and antimicrobial responses of local and infiltrating cells at the amputation site. The diversity and magnitude of immunological gene expression makes it difficult to measure the expression of key transcription factors and signaling molecules that are generally expressed at low levels. We show below that transcripts for key transcription factors and signaling molecules are robustly estimated using the axolotl tail amputation model. We used the model to screen 40 chemical inhibitors of major signaling pathway and described regenerative outcomes. We then used microarray analysis to explore one of the positive hits, a chemical inhibitor of WNT ligand secretion. Our results show that WNT signaling networks with multiple signaling pathways associated with development and regeneration. We detail our findings in the tables and figures below.

Table 1. Chemicals, targeted pathways, and experimental outcomes. Seven chemicals partially inhibited regeneration and six chemicals completely inhibited tail regeneration. All chemicals were tested at 10 uM unless otherwise noted.

Toxic

Pathway	Chemical
Wnt	Tideglusib
Wnt	TWS119
PI3K	Wortmannin
ATPase	Brefeldin A
ATPase	Blebbistatin
WNT/GSK3	BIO[3]

Complete Regeneration

Pathway	Chemical
Wnt	ICG-001
Wnt	KY02111
Wnt	Agonist II SKL 2001
TNFa	Lenalidomine
MMP	Doxycycline
Stat1	Fludarabine
Notch	Semagastat
Notch	MG-132
Hedgehog/ Smoothened	Vismodegib
FGFR	Danusertib
Apoptosis	NS3694
Translation	Azithromycin

Partial Regeneration

Pathway	Chemical
Wnt	XAV-939[1]
Wnt	Agonist I
Wnt	Nicotin
FGFR	BAZD4547
EGFR	Gefitinib
IL Receptor	Dexamethasone[2]
FGFR	SU-5402[5]

No Regeneration

Pathway	Chemical
Wnt	C59[4,5]
Wnt	IWR-1-endo
FGFR	BGJ398
TGFB/activin	SB-505124
IL6 inhibitor	Luteolin
V-ATPase H+ blocker	Concanamycin A[6]

[1] 40.0 uM; [2] 25.0 uM; [3] 15.0 uM; [4] 5.0 uM; [5] 2.5 uM; [6] 1.0 uM

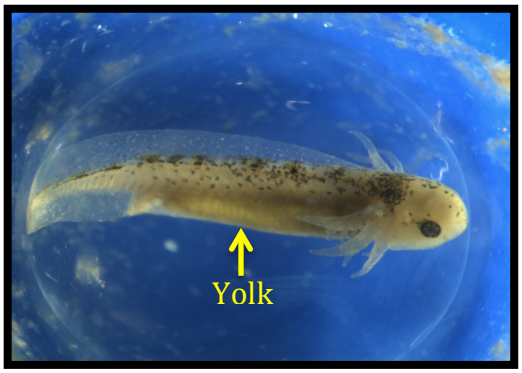

Figure 1A. Picture of a 1.0 cm axolotl embryo (Stage 39). At this stage, embryos can be manually removed from their egg membranes and reared individually in wells of microtiter plates. Thus hundreds to thousands of chemicals could be screened in a single experiment. Note the large yolk reserve in the gut that allows regeneration to be completed before the feeding stage is reached.

Figure 2. Axolotl tail regeneration assay. Axolotls embryos are hatched 1-2 days prematurely and 2 mm of the tail tip is removed with a sterile razorblade. Individuals are photographed before and after tail amputation, and then 5-7 days later. The left panel shows non-amputated, amputated, and regenerated tails. The right panel shows examples where chemical inhibitors completely blocked regeneration. For each inhibitor, no regeneration was observed 7 days post amputation (DPA).

Table 2. We performed a microarray experiment to identify genes that are differentially expressed as a result of blocking WNT ligand secretion using WNT-C59. The table below shows genes that were differently expressed at 48, 72, and 120 hrs post-amputation. The bolded genes were also significant at 168 hrs. These genes identify multiple signaling pathways (WNT, BMP, IL, NGF, MYCN, FGF, HOX, and EGF) associated with a normal regeneration response.

Probeset	Gene ID
axo01743-f_at	**APCDD1**
axo02472-f_at	**HAPLN3**
axo04593-f_at	**ETV4**
axo07001-f_at	**BMP2**
axo07256-f_at	**EPAS1**
axo07306-r_at	**IL8**
axo07473-f_at	**AREG**
axo07816-f_at	**FGF9**
axo08059-r_at	**LAMB1**
axo08282-f_at	**NGFR**
axo09045-f_at	**PHLDA2**
axo10046-f_at	**DYNC1I1**
axo11014-r_at	**MYCN**
axo12159-f_at	**SLC2A1**
axo13739-f_at	**ANKRD1**
axo13755-f_at	**DKK2**
axo18467-f_at	**DUSP6**
axo18474-f_at	**HOXC8**
axo23290-r_at	**unknown**
axo29791-f_at	**FGFR3**
axo29801-f_at	**AXIN1**
axo11014-r_at	**MYCN**
axo27228-f_at	**INHBB**
axo29536-f_at	**SP7**
axo29905-f_at	SP7
axo07974-f_at	INHBB
axo27128-f_at	WNT5A
axo28029-f_at	CYP26B1
axo01779-r_at	TMEM92
axo03170-f_at	PRICKLE2
axo07700-f_at	CTGF
axo07929-r_at	HMOX1
axo08144-f_at	MAS1
axo08178-f_at	MMP3
axo10448-r_at	LHX2

Figure 3. Examples of genes that were differentially expressed as a function of treatment (WNT-59 inhibitor is blue line, DMSO vehicle control is black line). The y-axis shows log2 expression and the x-axis shows time in hours post amputation. The early disruption of FGF9 transcription at 24 hours post-amputation suggests FGF9 is an early, direct target of WNT signaling. Note that FGF9 steadily increases in expression during normal regeneration. At 48 hrs, transcription of WNT (e.g. *dkk2*) and FGF pathway members is affected, and at 72 hrs, genes associated with retinoic acid signaling (*crabp2)* are transcriptionally altered. As tail outgrowth increases in rate, transcripts for negative regulators of cell proliferation decrease in abundance (e.g. *ctnna2*). Altered expression of *mrc1* at 168 hrs suggests phagocytic responses are not resolved when tail regeneration is inhibited. Many additional key developmental genes are significantly altered in the dataset, including *bmp2, hoxc8, hoxc10, hoxa3, hoxa13, pparg, axin, tgfb1, wif1, spry1, etv4, dll1, klf10, smad7, bmp7, id3, areg, ctgf, rarg, mmp3, mmp2, mmp1, cyr61, wnt5a, pax6, ngfr, adssl1, lep, ereg, fos, egr1, cdkn1b,* and many others.